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Isolation of a cDNA fragment coding for *Chlamydomonas reinhardtii* ferredoxin and expression of the recombinant protein in *Escherichia coli*

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A cDNA clone coding for mature *C. reinhardtii* ferredoxin has been isolated from a cDNA library using PCR and two oligonucleotide primers based on the N- and C-termini of the protein's amino acid sequence. The nucleotidic sequence of the PCR fragment (299 bp) agreed well with the amino acid sequence since a single conservative substitution (Thr-7 to Ser) could be deduced. The PCR fragment was inserted into the expression vector pTrc 99A, using the incorporated *Nco*I and *Bam*HI restriction sites and the construction used to transform *E. coli* (DH5α F'). After subsequent large scale expression and purification of the recombinant protein, biochemical and biophysical analysis have indicated that the product isolated from *E. coli* is homologous to native ferredoxin isolated from green algae.

Ferredoxin; PCR; *Chlamydomonas reinhardtii*

1. INTRODUCTION

Ferredoxins are small proteins containing iron-sulphur clusters which are implicated in the transfer of reducing power in many important biochemical reactions. Depending on their iron-sulfur content and EPR properties, they are normally classified into several families, one of which constitutes the 2Fe-2S soluble ferredoxins. This group includes plant ferredoxins, and also animal and bacterial ferredoxins such as adrenodoxin [1,2]. In plants ferredoxins function as electron donors for nitrite reductase, fatty acid desaturase, sulfite reductase, ferredoxin-dependent glutamate synthase and ferredoxin-thioredoxin reductase, the latter catalysing the light activation of several chloroplastic enzymes (see references in [3]). In plants and cyanobacteria they also function as intermediates of energy transfer in both cyclic and non-cyclic photophosphorylation [4], during which electrons pass from photosystem I, via ferredoxin, to either the cytochrome complex or NADP, a reaction catalysed by the thylakoid membrane-bound ferredoxin-NADP reductase [5]. Ferredoxin proteins are an integral part of the primitive mechanism of photosynthetic activity, common to all forms of photosynthesis in photosynthetic bacteria, algae, and higher plants. The determination of ferredoxin nucleotide and amino acid sequences is, therefore, of considerable interest for the study of the evolution of photosynthesis, and amino acid sequences of plant and algal ferredoxins have been used as the basis for at least one phylogenetic study [2,6,7].

The purification, properties and complete amino acid sequence of a soluble ferredoxin from the green alga, *Chlamydomonas reindhardtii*, have been reported previously, and its primary structure compared to those of ferredoxins from other plants and green algae [8]. In the present study two oligonucleotides, based on the N- and C-terminal amino acid sequences, were used to deduce the nucleotide sequence coding for the protein of this organism following PCR amplification. It is expected that this will provide further insight into the evolutionary relationships of ferredoxins, and also set the basis for the elucidation of the complete genomic nucleotide sequence.

Additionally, the ferredoxin nucleotide sequence has been used to transform *Escherichia coli*, allowing large scale expression of the protein. Initial studies on the properties of the recombinant protein are reported, and are compared to those of the native protein from *Chlamydomonas reinhardtii*.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, media, plasmids, manipulation of DNA and chemicals*

Escherichia coli strain DH5α F' was obtained from Gibco BRL. The expression plasmid pTrc-99a was a kind gift from Dr. Birgit Wetterauer. Bacteria were grown at 37°C on Luria-Bertani (LB) medium, supplemented with ampicillin (50 mg/ml) when the bacteria carried plasmids conferring resistance to this antibiotic. Isolation of

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plasmid DNA, preparation of DNA fragments, ligation and transformation of *E. coli* cells were carried out as described [9].

2.2. *Amplification of cDNA corresponding to the mature* C. reinhardtii *ferredoxin sequence by PCR*

Two oligonucleotides were constructed based on the previously determined amino acid sequence for *C. reinhardtii* ferredoxin [8], taking into account the coding preference of this alga [10]. The upstream 20-mer oligonucleotide was derived from the N-terminus protein sequence homologous to the non-coding strand, and included a *Nco*I restriction site (underlined) at the 5′ end, having the sequence:

5′ CC ATG GCC TAC AAG GTG ACC 3′.

Two C bases added between the *Nco*I site and the first tyrosine codon facilitated codon reading in the correct frame. The construction of the upstream primer thus resulted in the addition of an extra alanine (GCC) to the recombinant protein.

The downstream 24-mer oligonucleotide was derived from the C-terminus protein sequence complementary to the non-coding strand; this included a *Bam*HI restriction site (underlined) at the 5′ end immediately followed by a TTA codon (complementary to the TAA termination codon), with the sequence:

5′ GGA TCC TTA GTA CAG GGC CTC CTC 3′.

The size of the PCR product was therefore expected to be 299 base pairs (282 for ferredoxin itself, + 17 additional).

The PCR reaction was initiated directly with an aliquot of a cDNA library (a generous gift of J.D. Clermont Goldschmidt), used as template DNA as described in [11]. 4×10^8 clones were denatured by heating for 3 min at 97°C. The sample was then subjected to PCR in the presence of 400 nM of each oligonucleotide, 200 μM dNTPs, 1.3 mM Mg^{2+} and 2.5 units Taq polymerase (Beckman) (total volume 100 μl). 35 cycles (1 min 95°C, 2 min 58°C, 3 min 72°C) were performed, followed by an elongation of 6 min at 72°C.

2.3. *DNA sequencing*

An aliquot of the purified double-stranded PCR product having the predicted size (299 bp) was rendered blunt-ended by treatment with the Klenow fragment, and ligated into pBluescript SK$^+$ plasmid prepared previously by restriction with *Eco*RV. Following transformation of *E. coli* strain DH5α F′, the nucleotide sequence of the insert was determined by the dideoxy sequencing method using a Pharmacia T7 sequencing kit with SK and KS primers. The sequencing reaction was carried out at 45°C, which facilitated the reading of the sequence by reducing the number of secondary DNA structures, characteristic of *Chlamydomonas*-derived DNA.

2.4. *Expression of* C. reinhardtii *ferredoxin cDNA in* E. coli

An *Nco*I/*Bam*HI restriction fragment was digested from the sequencing vector, purified and ligated into the expression vector pTrc-99a [12]. The purified recombinant plasmid was then used to transform *E. coli* strain DH5α F′, one colony of which was used to initiate a 50 ml LB culture, supplemented with ampicillin. The culture was successively multiplied to a final volume of 4.8 litres over a period of 24 h and induced with 100 μM IPTG for the last 12 h. Cells were collected by centrifugation and stored frozen at −70°C before extraction of the protein.

2.5. *Extraction and purification of the recombinant protein*

Pelleted cells from the culture were resuspended in ice-cold buffer containing Tris-HCl (30 mM, pH 7.9), EDTA (1 mM), PMSF (500 μM), β-mercaptoethanol (1.4 mM) and benzamidine (500 μM). The suspension was disrupted by three passages through a precooled French pressure cell (Amicon), at 60 MPa. DNA was removed by the addition of streptomycin sulphate (0.3% w/v) followed by centrifugation for 30 min at 14,000 rpm. Thereafter, the protocol was similar to the one described previously [8]. All steps were performed at 4°C or on ice. Proteins in the crude extract were precipitated with ammonium sulphate, the fraction precipitating between 55% and 90% saturation being resuspended in approximately 20 ml buffer A (Tris-HCl, 30 mM, pH 7.9). This sample was loaded on to a Sephadex G50 column (60 × 5 cm) equilibrated with buffer A containing NaCl (200 mM) and proteins were eluted in 5 ml fractions by gravity flow. Fractions containing ferredoxin, which could be detected as a red band on the column, were pooled, dialysed against buffer A on an Amicon cell equipped with a YM 03 membrane and loaded on to a DEAE-Sephacel column (1 × 7 cm) equilibrated with buffer A. This column was washed with three volumes of buffer A, and proteins were then eluted in 5 ml fractions at a flow rate of 120 ml/h using a NaCl gradient from 0 to 0.8 M (25 ml, 25 ml). The elution of ferredoxin was detected by its absorbance at 420 nm, which normally occurred at approximately 0.4 M NaCl.

The ferredoxin fractions from the DEAE-Sephacel column were pooled and further purified by HPLC (see below), to remove contaminants absorbing at 260 nm. An elution peak was collected at 17.28 min. This sample was concentrated and dialysed against buffer A on an Amicon 'centricon 10 concentrator', and the sample was stored at −70°C.

2.6. *HPLC chromatography*

A mixture of the native and recombinant ferredoxin proteins was injected on a TSK Phenyl-5PW column, previously equilibrated with potassium phosphate buffer (0.1 M, pH 7.2), containing ammonium sulphate (1.8 M). Proteins were eluted over 40 min with a linear decreasing ammonium sulphate gradient (1.8 to 0 M) in the same buffer at a flow rate of 1 ml·min^{-1} and detected by their absorbance at 280 nm.

2.7. *Preparation of* C. reinhardtii *native ferredoxin and ferredoxin-NADP reductase (FNR)*

Ferredoxin was purified to homogeneity from *C. reinhardtii* cells as described previously [8]. FNR was purified from algal cells following a procedure similar to the one used for spinach FNR or pig ANR [3] which included affinity chromatographies on 2′,5′-ADP Sepharose and ferredoxin-Sepharose. The absorbance ratios (A_{456}/A_{275} and A_{456}/A_{380}) of the preparations were respectively 0.128 and 1.20, indicative of high purity.

2.8. *Ferredoxin assay*

Ferredoxin was assayed by monitoring the aerobic reduction of cytochrome *c* at 550 nm using an Uvikon spectrophotometer as described [3]. The reaction medium (1 ml) consisted of Tris-HCl (30 mM, pH 7.9), NADPH (0.15 mM), horse heart cytochrome *c* (0.04 mM), *C. reinhardtii* ferredoxin NADP-reductase (40 nM), and ferredoxin as required. Initial rates of cytochrome *c* reduction were calculated using a molar absorption coefficient of 15,300 $M^{-1} \cdot cm^{-1}$.

2.9. *Electron paramagnetic resonance analysis*

Recombinant or algal ferredoxins were dissolved in buffer A at a concentration of 100 μM, reduced by the addition of 10 mM dithionite, and immediately frozen in EPR tubes in liquid nitrogen. Spectra were recorded at 40K on a Bruker ESR 200 spectrometer equipped with an Oxford Instruments cryostat. Conditions for measurements were: power 20 mW, gain 5×10^4, modulation 10 gauss, frequency 9.428 GHz.

3. RESULTS

3.1. *Cloning and sequencing of* C. reinhardtii *ferredoxin cDNA nucleotidic sequence*

The availability of the complete amino acid sequence of *C. reinhardtii* ferredoxin has allowed us to isolate a cDNA fragment coding for the mature protein, using PCR and two synthetic oligonucleotides. The insertion of two nucleotides into the N-terminus primer, between

the *Nco*I sequence and the start of the first ferredoxin codon, achieved the correct reading frame for the protein after insertion into the expression vector. The decision that the extra codon so formed should be that of alanine was prompted by the occurrence of this amino acid at the initial N-terminus position in the majority of plant and algal ferredoxins analysed to date (see Fig. 6 in [8]). This technique, which has already proved successful for *C. reinhardtii* Ch2 [11], allowed not only the rapid sequencing of the fragment after cloning into the Bluescript vector, but also the expression of the functional protein.

As indicated by the derived amino acid sequence (Fig. 1), only one conservative substitution was observed compared to the 94-residue amino acid sequence previously published for ferredoxin [8], the threonine at position 7 in this latter sequence being replaced by serine. It is not yet clear whether this single amino acid difference reflects the existence of variants of ferredoxin in *C. reinhardtii*. In connection with this possibility, it is of interest that at least two variants have already been reported for spinach leaf ferredoxin [13]. Alternatively, the difference could result from an error in the PCR reaction. This hypothesis can be checked by subsequent cloning and analysis of cDNA clones obtained from the library without PCR amplification.

Of the 94 codon triplets, 11 were found to be different from the documented *C. reinhardtii* codon preference [10]. Of these eleven, six concerned codons for alanine which all had the sequence GCT. As the whole protein sequence contained 13 alanine residues, one of which was already specified by the N-terminus oligonucleotide, it appears that for this *C. reinhardtii* ferredoxin sequence, GCT is used as readily as GCC as a codon for this amino acid.

```
                                1
CCATG GCC TAC AAG GTG  ACC  CTG  AAG TCC
NcoI A   Y   K   V    T    L    K   S
                   10
CCT TCG GGT GAC AAG ACC ATT GAG TGC CCC
P*  S   G   D   K   T   I*  E   C   P

GCT GAC ACC TAC ATC CTG GAC GCT GCT GAG
A*  D   T   Y   I   L   D   A*  A*  E

GAG GCC GGC CTG GAC CTG CCC TAC TCT TGC
E   A   G   L   D   L   P   Y   S*  C

CGC GCT GGT GCT TGC TCC AGC TGC GCC GGC
R   A*  G   A*  C   S   S   C   A   G

AAG GTC GCT GCC GGC ACC GTC GAC CAG TCG
K   V   A*  A   G   T   V   D   Q   S*

GAC CAG TCC TTC CTG GAC GAT GCC CAG ATG
D   Q   S   F   L   D   D   A   Q   M

GGC AAG GGC TTC GTG CTG ACC TGC GTG GCC
G   K   G   F   V   L   T   C   V   A

TAC CCC ACC TCG GAC TGC ACC ATC CAG ACC
Y   P   T   S*  D   C   T   I   Q   T

CAC CAG GAG GAG GCC CTG TAC TAA GGATCC
H   Q   E   E   A   L   Y  Ter BamHI
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Fig. 1. Nucleotidic acid sequence coding for *Chlamydomonas reinhardtii* ferredoxin. The isolated and purified PCR fragment of 299 base pairs (282 for ferredoxin itself, + 17 additional) was sequenced after being inserted into pBluescript SK$^+$. Underlined regions were coded by the original PCR primers; * indicates codons differing from those preferred by *Chlamydomonas*.

3.2. *Expression in* E. coli *of recombinant* C. reinhardtii *ferredoxin*

The *Nco*I and *Bam*HI sites in the PCR product were used to ressect the fragment into expression vector pTrc 99A and the resulting construction was used to transform *E. coli* DH5α F′. Following purification of the expressed protein, approximately 1 mg of homogeneous protein was obtained from 30 g (initial wet weight) of *E. coli* cells. Based on the cytochrome *c* reduction assay, we estimate that the yield of the purification procedure was approximately 50% (data not shown). The addition of 100 μM $FeSO_4$ to the *E. coli* culture medium was not found to increase the expression of the native protein (data not shown). In addition, the use of a different expression system (the pET vector in conjunction with *E. coli* strain BL21) was also not found to increase expression (data not shown). In parallel experiments we have used the same vectors to produce *C. reinhardtii* thioredoxin and found that the expression was greatly enhanced when using the pET vector system (unpublished data). We therefore conclude that the lack of increase in expression of the recombinant ferredoxin is not due to an incompatibility of the *C. reinhardtii* nucleotidic sequence. A possible explanation is the existence of a rate-limiting step during assembly of the iron-sulfur centre in *E. coli*. In relation to this, recombinant ferredoxins from other sources have already been expressed in *E. coli* [14,15], but it is not always clear from these papers what the level of expression is. Nevertheless, the level of expression of the recombinant ferredoxin reported in the present work is approximately ten times higher than that of the endogenous protein as previously determined [16]. This fact in itself supports the spectral and sequence data (see below) showing that the isolated product is indeed a recombinant *C. reinhardtii* ferredoxin, and not the native 2Fe-2S ferredoxin from *E. coli*.

3.3. *EPR and absorption spectra*

After reduction with dithionite, EPR spectra of both algal and recombinant *Chlamydomonas reinhardtii* ferredoxins (Fig. 2A) show a very characteristic rhombic signal belonging to the g_{av} 1.96 family, with *g* values of 2.060, 1.970 and 1.893. This is, therefore, further confirmation that the recombinant product isolated from *E. coli* is homologous to the native protein isolated from the green algae. In addition, these signals clearly indicate that the recombinant protein belongs to the family of 2Fe-2S plant type ferredoxins (for example, spinach ferredoxin shows similar *g* values of 2.04, 1.96 and 1.89 [17]). The signal is very different from the one recorded for *E. coli* 2Fe-2S 'adrenodoxin type' ferredoxin [16] which exhibits an axial type signal.

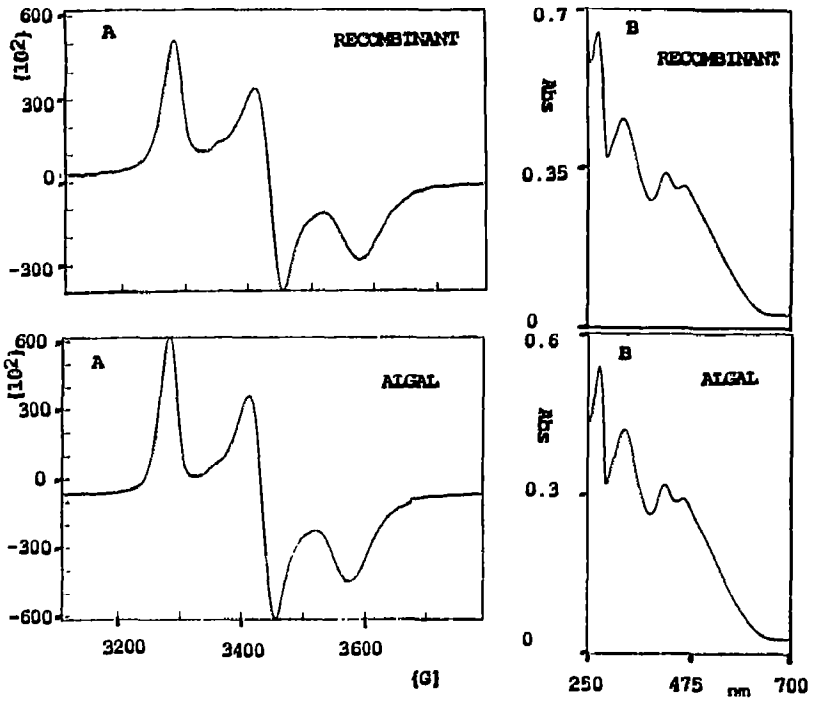

Fig. 2. (A and B). EPR and absorption spectra of algal or recombinant *Chlamydomonas reinhardtii* ferredoxin. (A) EPR spectrum of algal or recombinant protein following reduction with 5 mM dithionite. The protein concentration was approximately 1 mg/ml, i.e. 100 μM (in Tris-HCl 30 mM, pH 7.9). (B) absorption spectrum of oxidized algal or recombinant ferredoxin. The protein concentration was approximately 0.4 mg/ml, i.e. 40 μM (in Tris-HCl 30 mM, pH 7.9).

The absorption spectra of the proteins (Fig. 2B) show broad absorption bands with maxima at 277, 330, 422 and 467 nm. The A_{422}/A_{277} absorption ratio can be as high as 0.54 which is also indicative of a high homogeneity for the preparation. The recombinant spectrum differs from that of the *E. coli* 2Fe-2S endogenous ferredoxin (absorption maxima at 277, 325, 416 and 460 nm) [16], the major differences being the shape of the peak in the UV absorbance range, and the position of the band at 422 nm, which replaces a band at 416 nm in the *E. coli* protein.

3.4. *Chromatographic properties and N-terminus amino acid sequence analysis*

The purity of the recombinant ferredoxin preparation, as well as its identity with *C. reinhardtii* ferredoxin, were further confirmed by analytical HPLC chromatography (Fig. 3). Either chromatographed alone or with the native algal ferredoxin on a hydrophobic interaction column, a single symmetric peak was observed on Phenyl TSK with a retention time of 18.25 min (under identical conditions spinach leaf ferredoxin could be resolved into its two isoforms [13], with retention times of 10.6 and 12 min, respectively). The fact that this technique, which separates proteins on the basis of their hydrophobic interactions, did not detect the two amino acid differences between the recombinant and native *C. reinhardtii* ferredoxins suggests that the modifications have not changed the overall hydrophobic behavior of the molecule. This result was actually expected because of the nature of the biochemical modifications.

An N-terminus amino acid sequence analysis of recombinant ferredoxin was performed: no heterogeneity could be detected and the results were identical to the predicted sequence:

Ala-Tyr-Lys-Val-Thr-Leu-Lys-Ser.

As expected, the threonine residue in position 7 of the sequence published in [8] was replaced by a Ser residue. In addition, the sequence was completely different from the one published for the adrenodoxin type *E. coli* ferredoxin [16], this being:

Pro-Lys-Ile-Val-Ile-Leu-Tyr.

3.5. *Biochemical reactivity*

The results from the cytochrome *c* reduction assay (Fig. 4) indicate that *C. reinhardtii* ferredoxin NADP reductase (FNR) recognizes the algal and recombinant ferredoxins with exactly the same efficiency, despite the two amino acid differences between the two proteins. The observed saturation kinetics are in good agreement with the functioning of a homologous FNR/ferredoxin

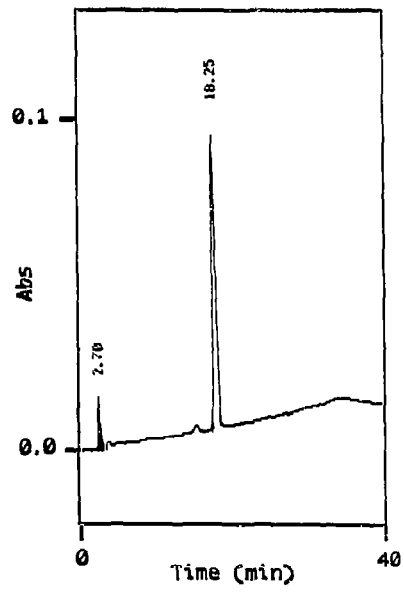

Fig. 3. HPLC analysis of native and recombinant *C. reinhardtii* ferredoxins. A mixture of native and recombinant proteins (10 μg each) was injected onto a TSK Phenyl-5PW column, equilibrated with K^+-phosphate buffer (0.1 M, pH 7.2). Proteins were eluted with a decreasing ammonium sulphate gradient (1.8 to 0 M) over 40 min at a flow rate of 1 ml/min.

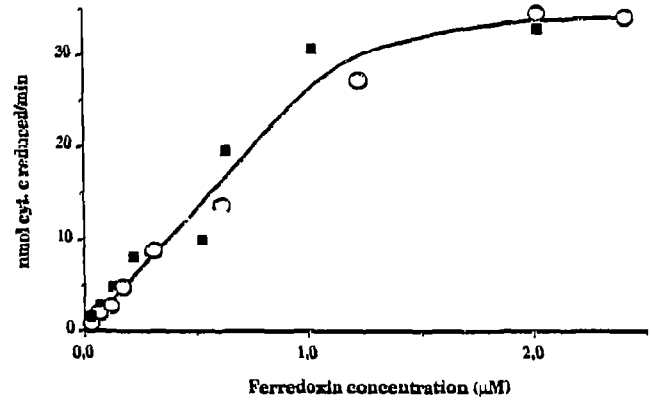

Fig. 4. Ferredoxin-dependent reduction of cytochrome *c* by ferredoxin-NADP reductase. Ferredoxin-NADP reductase concentration in the cuvettes was 40 nM. Cytochrome *c* reduction was followed at 550 nm at 30°C (■, native ferredoxin protein; ○, recombinant protein).

system, a different type of ferredoxin such as adrenodoxin being required at a much higher concentration [3].

4. DISCUSSION

The results presented in this paper provide the first nucleotidic sequence for *C. reinhardtii* ferredoxin. As already stated in the Introduction, this sequence can be used as an evolutionary tool provided one keeps in mind that 34 out of the 299 base pairs are encoded by the oligonucleotides. Thus, only the 255 base pair long nucleotidic sequence, coding for 85 amino acids (from Leu-5 to Gln-89 e.g. about 90% of the total 94 amino acids), can be used for this purpose. A first important conclusion can be drawn after looking at the ferredoxin sequence: the coding bias for the amino acid Ala certainly does not conform to the one previously published for *C. reinhardtii* [10]. From this nuclear encoded sequence, it is very clear that both GCT and GCC (and not only GCC) are equally often used for coding Ala and this should be taken into account for designing other oligonucleotides.

The comparison of the deduced amino acid sequence from Leu-5 to Gln-89 with the one obtained by direct amino acid sequencing shows that they are nearly identical with a single modification (Thr-7 replaced by Ser). Thus, this sequence is extremely valuable in validating the one formerly obtained by direct amino acid sequencing. Of course, there are plenty of examples where the establishment of a nucleotidic sequence has led to correct a wrong amino acid sequence and vice versa, and thus it is certainly worth obtaining both pieces of information. In addition, in this particular case, it raises the interesting possibility of the existence of variants for ferredoxin in green algae. Whether these variants can exist in a single species of *C. reinhardtii* or whether they result from using slightly different strains remains of course to be proven.

The biochemical studies in this paper indicate that the isolated recombinant ferredoxin is homologous to algal ferredoxin and different from adrenodoxin type *E. coli* ferredoxin. This conclusion is based on three different set of results: EPR and UV/visible absorption spectra and N-terminus amino acid sequencing. EPR spectra were not available for *C. reinhardtii* ferredoxin so far and hence they bring additional valuable information about the similarity between green algae and higher plant 2Fe-2S soluble ferredoxins. Another observation is that the two amino acid differences (extra Ala and Thr-7 replaced by Ser) have not significantly changed the chromatographic and spectral behavior of the protein. In this respect, it is interesting to mention that the validity of both sequences has been fully confirmed by electrospray mass spectrometry (data not shown).

In principle, ferredoxin is nuclear encoded [18,19], but since we cloned the fragment devoid of the transit peptide sequence with PCR, we did not have any problem with the processing of the protein. Additionally, the purification procedure indicates that at least part of the protein is present in the soluble fraction and reassociated with the iron sulfur centre. It is concluded that *E. coli* cells are able to express the *C. reinhardtii* nucleotidic sequence and to reassemble the iron sulfur centre together with the polypeptide. The availability of such an expression system for eucaryotic green algal ferredoxin opens the way to site directed mutagenesis studies espe-

cially at the level of the cysteine residues linking the iron sulfur cluster [20].

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